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BACTERIAL AND PHYTOPLASMA DISEASES

Winter habitat of *Xylophilus ampelinus*, the cause of bacterial blight of grapevine, in Japan

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Abstract *Xylophilus ampelinus*, the cause of bacterial blight of grapevine, was first identified in 2009 in Hokkaido, Japan. We investigated the distribution of *X. ampelinus* in grapevines and its winter habitat in Hokkaido. After defoliation of artificially infected potted plants, new branches were cut and stored in plastic bags at 5 °C in the dark for 60 days. PCR analysis of samples from the stored branches using a pathogen-specific primer set detected *X. ampelinus* in the axillary buds but not in the epidermis and vascular bundle. In the naturally infected vineyards, we confirmed the presence of *X. ampelinus* in the axillary buds and in the vascular bundles at defoliation. However, the pathogen DNA was not detected in the vascular bundles or sap after overwintering. In contrast, the detection frequency in whole axillary buds decreased only slightly, even after overwintering. Additionally, the bacteria were detected in cold-stored bracts and wool at a higher frequency than in the interior axillary bud tissues. Our findings indicate that *X. ampelinus* likely is not maintained inside tissues or sap and that it might not survive on plant surfaces; rather, bacteria can be found on the underside surface of the bract and bud wool during winter in Hokkaido.

Keywords *Xylophilus ampelinus* · Bacterial blight · Grapevine · Overwintering · Bud

Introduction

Xylophilus ampelinus (Panagopoulos 1969) (Willems et al. 1987) causes bacterial blight of grapevine in its only known host, *Vitis vinifera* (Panagopoulos 1980). Symptoms include cracks and cankers along shoots, discoloration of xylem tissues, angular necrotic lesions on leaves, and death of the infected canes and branches (Lopez et al. 1981; Ridé and Marcelin 1983). Severe infection can lead to serious harvest losses, because the infected branch is usually destroyed. Unfortunately, chemical agents do not aid in disease control (Panagopoulos 1987).

In Japan, the first outbreak of the disease occurred in Hokkaido Prefecture in 2009 (Shinmura et al. 2012), and diseased plants were distributed throughout the vineyard. The symptoms were originally confused with the dead arm of grapevine caused by *Phomopsis viticola*, a fungal pathogen that is abundant in Hokkaido. Therefore, almost all grape producers applied fungicides, that failed to control the disease, leading to the discovery of the causal bacterium (Shinmura et al. 2012).

The disease is associated with cool and moist conditions. The causative bacterium is transmitted via moist wind, rain, and sprinkled irrigation (Bradbury 1991). Evidence also indicates that the bacteria are mechanically transmitted via shear blades during pruning and harvesting (Ridé et al. 1977). Indeed, the bacterium was isolated from pruning shears and harvesting machine blowers in France (Ridé 2000). Therefore, bleeding sap is likely an important source of contamination. However, contamination was not replicated using an experimental pruning procedure in autumn in Hokkaido (T. Komatsu, unpublished data). The aim of the present study was to examine the ecology of *X. ampelinus* in Hokkaido vineyards by determining the bacterial distribution within specific plant tissues after

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defoliation and overwintering in the vineyard and within detached branches after storage.

Materials and methods

Artificial inoculation

In March 2012, the branches of three primary winemaking grape varieties, Kerner, Zweigeltrebe and Seibel 5279, growing in the vineyard of the Central Agricultural Experiment Station, Hokkaido Research Organization were cut from healthy grapevines. Each branch was planted in a plastic pot (12 cm diameter \times 15 cm high) filled with Kanuma soil (a weathered pumice) and horticultural soil (Takii & Co., Kyoto, Japan) mixed at a 1:1 ratio (v/v). Branches were grown in a greenhouse at the Experimental Station. In May 2012, 40 pots per cultivar of plants that had grown roots and new branches were moved to an enclosure (3 m high \times 15 m long \times 5.4 m wide) covered with a water-permeable shade (50 % reduction of far infrared light, 15 m long \times 8 m wide; Megacool, Mitsubishi Plastics Agri Dream Co., Tokyo, Japan).

Xylophilus ampelinus strains BB-7 and BB-9 (isolated from Kerner and Zweigeltrebe, respectively, in Yoichi, Hokkaido in 2009) were shake-cultured in 100 mL of nutrient broth (10 g beef extract, 10 g bacto-peptone, and 3 g NaCl/1,000 mL, pH 7.2) at 125 rpm and 25 °C for 7 days. The two bacterial suspensions were mixed and diluted with distilled water to 1,000 mL, and the bacterial suspension (2 mL) was applied to each leaf of 30 potted grape plants using a hand sprayer on five consecutive days at three times: June 8–12, July 9–13, and August 24–28, 2012. Suspensions of 1.09×10^7 colony forming units (cfu)/mL, 6.74×10^9 cfu/mL, and 9.67×10^9 cfu/mL were prepared just before the inoculation. Ten potted grape plants were sprayed with the same amount of water without inoculum as controls. After inoculation, the plants were covered with a plastic sheet (0.9 m long \times 1.8 m wide) until the next morning to maintain high humidity. Potted grape plants were watered by sprinkling the soil and the leaf surface daily with water in the morning if it was not raining.

The incidence of diseased leaves was assessed on October 3, 2012. After defoliation on November 20, almost all individuals had one new branch with multiple buds; each new branch was cut off, leaving two buds on the grape plant, and the branches were stored in plastic bags at 5 °C in the dark for 60 days. The presence of *X. ampelinus* in the axillary buds, epidermis, and vascular bundles was determined in triplicates of 15 branches of each cultivar.

Natural infection

To determine the bacterial distribution in grapevines, plant samples were collected from vineyards 1 (Kerner in Yoichi), 2 (Zweigeltrebe in Furano, Hokkaido), and 3 (New York Muscat in Furano). The disease frequencies recorded for the leaves were 1.3 and 3.7 % in vineyards 1 and 2 (September 2012), respectively, and 9.8 % in vineyard 3 (September 2013). In vineyard 3, 20 samples of axillary buds and vascular bundles from 60 pruned branches of 20 trees were collected after all the leaves had fallen in October 2013. After overwintering, one sample each of the axillary buds, sap, and vascular bundles was collected per tree before sprouting from the end of April to the beginning of May 2013 from vineyards 1 and 2 and from the end of April 2014 from vineyard 3. The first leaves that developed at the bases of the branches and the spikes were collected as one sample per tree from the beginning to middle of June 2013 from vineyards 1 and 2 and from the end of May to the middle of June 2014 from vineyard 3. Further, the remaining branches collected from vineyard 3 in October 2013 were stored in a plastic bag at 5 °C in the dark. The axillary buds were classified into bract, wool, and tissues inside axillary buds and were analyzed after storage for 50 days. The 20 samples were analyzed for the presence of *X. ampelinus*. These experiments were conducted in triplicate.

Asymptomatic samples

In August 2013, leaves were collected as one sample per tree from the five commercial vineyards in Yoichi as follows: vineyards 4–7 with cultivated Kerner and 8 with cultivated Kerner and Sauvignon Blanc. All vineyards were highly infected in 2009 and 2010, whereas all of the plants in these vineyards were asymptomatic in 2013. Twenty shoots (approximately 20 cm long) were collected from plants from each of the vineyards to analyze for DNA of *X. ampelinus*.

DNA extraction and PCR analysis

To extract DNA from the vascular bundle tissue, the epidermis and cortex were scraped off with a sterilized razor blade, and a 10-mm length of the stele was excised and immersed in 100 μ L of TE buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.0) to soften it. The stele was then ground using sand paper (#100, Holt Production Ltd., UK) according to the method of Nakaune and Nakano (2006). This paste of macerated tissue was added to 400 μ L of AP1 buffer from the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and placed into a new microcentrifuge tube. Three layers of epidermis (10 mm long \times 2 mm wide), three buds, a 1-cm² section of leaf, and a 5-mm spur were

softened separately in 100 μL of TE buffer. The respective samples were then homogenized with a sterilized mortar and pestle. Immediately after the branches were pruned in April 2013 and 2014, approximately 1.0 mL of the oozing sap was collected in 1.5 mL microcentrifuge tubes from each cut surface. In 2013, to culture any bacteria in the sap, 100 μL of sap was mixed with 900 μL of nutrient broth in a 2 mL tube and incubated at 25 °C with shaking at 125 rpm for 7 days. The broth was then plated on King's B medium (Eiken Chemical Co., Tochigi, Japan) and incubated at 25 °C. After 10 days of incubation, the morphology of the bacterial colonies was recorded, and 10 colonies were isolated with sterilized toothpicks and suspended in 50 μL of sterilized distilled water. The suspension was heated at 95 °C for 10 min and then cooled rapidly for 5 min on ice; after centrifugation, the supernatant was used in PCR analysis as described in the next paragraph. In addition to bacterial culture supernatant, in 2014, intact sap was obtained without pre-cultivation to detect bacteria using PCR.

DNA was extracted from plant organs and intact sap using the DNeasy Plant Mini Kit according to the manufacturer's instructions. Amplification reactions included 1 μL of DNA and 24 μL of reaction mixture: 1 μM of each primer (XaTS1; 5'-TGC GTAGTTCAACACCAAAGT-3', XaTS2; 5'-TATGACCTCTTTCCACCAGC-3'), 1.25 U of *ExTaq* DNA polymerase (Takara Bio, Shiga, Japan), 200 μM dNTP mixture, and 1 \times PCR buffer with MgCl_2 . Amplification conditions were as follows: initial denaturation at 94 °C for 5 min; 40 cycles at 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 45 s; and a final extension at 72 °C for 8 min (Manceau et al. 2005). To prove that there was no interference of inhibitory substances in the extracts of the buds and vascular bundles, 20 DNA samples were highly purified with Mag Extractor Plant Genome (Toyobo Co., Tokyo, Japan), which is effective for purifying nucleic acids extracted from soil (Kageyama et al. 2003), and the aforementioned PCR method was repeated. PCR products were subjected to agarose gel (1.5 % w/v) electrophoresis for 30 min at 100 V, and the amplicons were detected by staining the gels with 0.01 % (v/v) Gel Red (Wako Pure Chemical Industries, Tokyo, Japan).

Results

Artificial inoculation

Potted grapevine plants inoculated with *X. ampelinus* exhibited the typical symptoms of bacterial blight on every branch. We examined all leaves and counted diseased leaves. Kerner had 405 diseased leaves (163 healthy), Seibel5279 had 619 diseased (172 healthy), and Zweigeltrebe

had 567 (172 healthy). The frequency of diseased leaves, in order of most frequent, was thus 23.8 % (SE \pm 2.8) on Seibel 5279, 21.7 % (SE \pm 11.8) on Kerner, and 17.7 % (SE \pm 3.5) on Zweigeltrebe in September 2012. The analysis of cut branches stored at 5 °C for 60 days revealed the presence of *X. ampelinus* in 100 % of the axillary buds of Kerner and Zweigeltrebe and in 80 % of Seibel 5279, but not in the epidermis or vascular bundle (Table 1; Fig. 1). Symptoms and pathogen-specific DNA were not detected in the controls inoculated with water.

Natural infection

In plants with a high incidence of diseased leaves in vineyard 3 in 2013, the presence of *X. ampelinus* was detected in 100 % of whole axillary buds and vascular bundles immediately after defoliation (Table 2; Fig. 2a). The whole axillary buds had a high number of positive reactions for *X. ampelinus*-specific DNA in the spring of 2013 (21.7 % in vineyard 1 and 40.0 % in vineyard 2) and 2014 (63.3 % in vineyard 3; Table 2; Fig. 2b). We observed a few bacterial colonies on the plates inoculated with incubated sap in 2013, but none had the characteristic features of *X. ampelinus*, e.g., non-mucoid, smooth, yellow, round, and fully circular colonies. Further, DNA specific for *X. ampelinus* was not detectable by PCR amplification from any colony or from intact sap collected in 2014 from vineyard 3 (Table 2; Fig. 2c), nor from the vascular bundles after overwintering (Table 2; Fig. 2d). In contrast, the DNA frequency in the whole axillary bud only slightly decreased after overwintering (Table 2; Fig. 2b). Although the first leaves that developed at the bases of the branches and spikes were asymptomatic, they were positive for DNA of *X. ampelinus* (Table 2; Fig. 2e). In addition, similar amplification patterns were observed for DNA samples prepared using only the DNeasy Plant Mini Kit versus using the highly purified DNA samples (data not shown).

The frequency of DNA of *X. ampelinus* in the cold-stored bract and wool segments of the axillary bud was particularly high at 96.7 %. The DNA was detected even in the tissues inside axillary buds, but only in 23.3 % of the tested tissues.

Detection of *X. ampelinus* from asymptomatic plants

In 2013, pathogen-specific DNA was detected in 5–30 % of the leaves collected from asymptomatic plants, which were diseased in 2009 (Table 3).

Discussion

Xylophilus ampelinus grows very slowly on artificial media and forms visible colonies only after 10 days of

Table 1 Detection of *Xylophilus ampelinus* in artificially inoculated branches removed from potted *Vitis vinifera*

| Inoculation ^a | Cultivar | Disease leaves (%) ^b | PCR detection (%) | | |
|--------------------------|--------------|---------------------------------|-------------------------------|-----------|------------------|
| | | | 60 days after storage at 5 °C | | |
| | | Before defoliation | Buds | Epidermis | Vascular bundles |
| Inoculated | Kerner | 21.7 | 100 | 0 | 0 |
| | Seibel 5279 | 23.8 | 80 | 0 | 0 |
| | Zweigeltrebe | 17.7 | 100 | 0 | 0 |
| Uninoculated | Kerner | 0 | 0 | 0 | 0 |
| | Seibel 5279 | 0 | 0 | 0 | 0 |
| | Zweigeltrebe | 0 | 0 | 0 | 0 |

^a Potted *V. vinifera* (30) were inoculated by spraying with *X. ampelinus* strain BB-7 and BB-9 suspensions, and 10 potted *V. vinifera* served as Controls (uninoculated) and were sprayed with water

^b Percentage of diseased leaves of total leaves analyzed. Total leaf numbers: inoculated- Kerner, 405; Seibel 5279, 619; and Zweigeltrebe, 567. Total leaf numbers: uninoculated- Kerner, 163; Seibel 5279, 172; Zweigeltrebe, 172

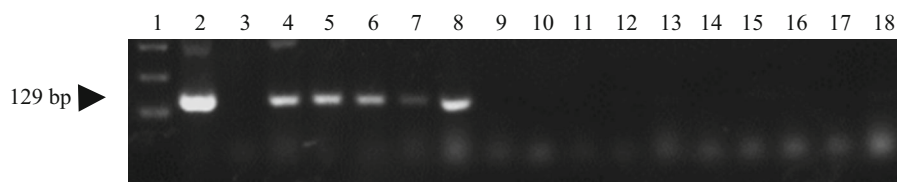


Fig. 1 PCR detection of *Xylophilus ampelinus* in detached, inoculated branches of grapevine (Kerner) stored at 5 °C for 60 days. *Lanes: 1* 100 bp ladder; *2* *X. ampelinus* (129 bp, positive control); *3* sterile distilled water (negative control); *4–8* buds; *9–13* epidermis; *14–18* vascular bundles

Table 2 Detection of *Xylophilus ampelinus* in the tissues of naturally infected *Vitis vinifera* in commercial vineyards

| Vineyard number | PCR detection rate (%) | | | | | | |
|-----------------|------------------------|-----------------|----------------------------------|------|------------------|--------|--------|
| | After defoliation | | After overwintering ^a | | | | |
| | Bud | Vascular bundle | Buds | Saps | Vascular bundles | Leaves | Spikes |
| 1 | n.t. | n.t. | 21.7 | 0 | 0 | 0 | 30 |
| 2 | n.t. | n.t. | 40.0 | 0 | 0 | 45 | 85 |
| 3 | 100 | 100 | 63.3 | 0 | 0 | 70 | 70 |

Plant samples were collected from vineyard 3 just after defoliation in October 2013

n.t. not tested

^a Plant samples were collected in 2013 from vineyard 1 and 2 and in 2014 from vineyard 3. The buds, vascular bundles, and sap were collected before sprouting

culture (Bradbury 1991). Furthermore, because selective medium is not available, saprophytic bacteria may easily overgrow the pathogen. Therefore, we examined samples using PCR with primers that were developed by Manceau et al. (2005) specifically to amplify the DNA of *X. ampelinus*. Amplification of the specific DNA from grapevine tissues is generally difficult because of the high concentrations of inhibitory substances in plant extracts (Minsavage et al. 1994; Rowhani et al. 1993). However,

we observed similar amplification patterns for DNA samples prepared using only a DNA extraction kit and for highly purified DNA samples. Therefore, we concluded that the DNA samples prepared from grapevine tissues by using the extraction kit were suitable to detect *X. ampelinus*. Additionally, any negative results could not be attributed to the presence of inhibitors in the DNA preparation because we used improved DNA extraction technology.

Fig. 2 PCR detection of *Xylophilus ampelinus* in naturally infected grapevines in vineyard 3. **Panels:** **a** buds (lanes 4–13) and vascular bundles (lanes 14–23) collected just after defoliation in 2013; **b** bud (lanes 4–23); **c** sap (lanes 4–23); **d** vascular bundle (lanes 4–23); **e** leaves (lanes 4–13) and spikes (lanes 14–23) collected after overwintering in 2014. All panels, lanes: 1 100 bp ladder; 2 *X. ampelinus* (129 bp, positive control); and 3 sterile distilled water (negative control)

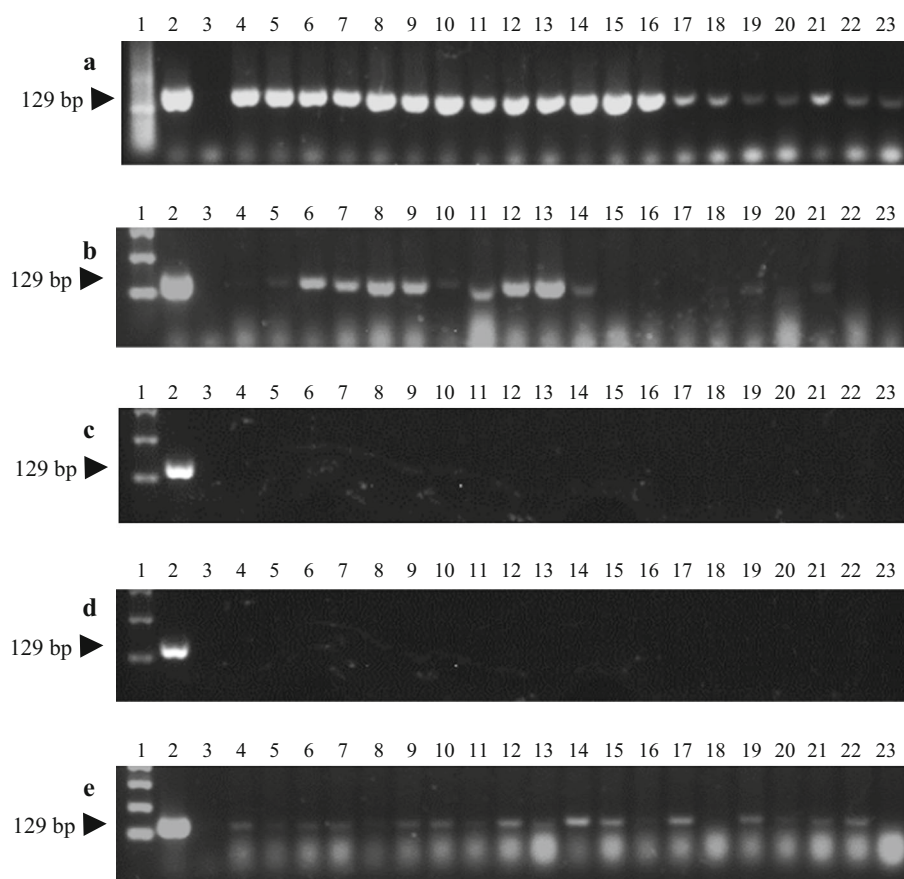


Table 3 Detection of *Xylophilus ampelinus* in asymptomatic leaves from commercial vineyard in Yoichi in 2013

| Vineyard number | Cultivar | PCR detection rate (%) |
|-----------------|-----------------|------------------------|
| 4 | Kerner | 15 |
| 5 | Kerner | 10 |
| 6 | Kerner | 5 |
| 7 | Kerner | 15 |
| 8 | Kerner | 10 |
| 8 | Sauvignon Blanc | 30 |

To determine the distribution of *X. ampelinus* within grapevines, we analyzed infected plants for the presence of pathogen-specific DNA after the growing season. In France, *X. ampelinus* was found in the sap and old vascular bundles (Grall et al. 2005). In contrast, in Hokkaido, we detected the bacterium only in the axillary buds in the spring before sprouting in the highly infected vineyard; however, *X. ampelinus* DNA was detected in the vascular bundle and in buds immediately after defoliation in autumn (Table 2; Fig. 2a). These results indicate that, in Hokkaido, *X. ampelinus* likely hibernates in the bud rather than in the sap or the vascular bundle. The northern European varieties

that are mainly cultivated in Hokkaido differ from those cultivated in France, and *X. ampelinus* is not the causal agent in northern Europe (OEPP/EPPO 2013; <https://www.eppo.int/QUARANTINE/listA2.htm>). Further, Hokkaido is located in a subarctic zone where the average temperature is below freezing for approximately 4 months (from the last half of November to the first half of March). Therefore, we think that the difference in bacterial strains and/or winter temperatures determines which part of the plant the bacterium inhabits during latency.

The bacterium spreads by wind and rain in Europe (Panagopoulos 1987). The bacterium most likely also spreads in Hokkaido via these modes because potted healthy grapevines were infected after they were placed between diseased plants (T. Komatsu, unpublished data). Moreover, the bacteria were detected in the bracts and wool at a higher frequency than in the tissues inside axillary buds. Grall and Manceau (2003) found that, although bacteria primarily formed colonies in the vascular bundles after scratch inoculations, the bacteria did not spread to the new shoot. This observation indicates that it is difficult for *X. ampelinus* to migrate through plant tissues, leading us to conclude that the bacteria are transported mainly along the surface of the vine by water or wind to the axillary buds

without entering the plant. The bacterium was not detected on the epidermis of branches that were refrigerated (Table 1), indicating that the bacterium might not survive on the plant surface during winter. Therefore, we surmised that only the bacteria inhabiting the underside surface of the bracts and wool survive the winter.

The latent period of *X. ampelinus* depends on many factors such as climate (Ridé and Marcelin 1983), and the bacterium can survive for several years on the grapevine without inducing symptoms. Therefore, the bacteria are likely to begin to proliferate when the humidity and temperature increase to levels that support growth (Grall and Manceau 2003). Here, we showed that bacterial DNA was detected even in leaves with no symptoms that were collected from a vineyard throughout the 2013 growing season (Table 3). In Hokkaido, the bacterium was first identified in 2009 (Shinmura et al. 2012); however, the plants may have been infected several years earlier, with the disease becoming more severe over time in vineyards that were latently infected. Because the average amount of rain was higher during the summer of 2009 and the temperatures were lower than normal, a burst of bacterial growth may have suddenly induced symptoms. Although recent incidence of the disease has been low, the bacteria may have spread more widely, thereby increasing the potential for future outbreaks. Our findings indicate that it is necessary to monitor bacterial distribution in various vineyards so that latent bacteria can be detected and preventive methods deployed to avoid an outbreak.

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